PERIODICUM BIOLOGORUM
VOL. 120, No 2–3, 105–110, 2018
DOI: 10.18054/pb.v120i2-3.6866

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

original research article

Combined zinc and nitrogen applications at panicle initiation for zinc biofortification in rice

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Keywords: alkaline soil; foliar fertilisation; grain zinc density

Received May 06, 2018.
Revised November 03, 2018.
Accepted November 03, 2018.

Abstract

Background and purpose: *Increasing zinc (Zn) concentration in rice grains can help improve Zn nutrition of people. The combinations of Zn and nitrogen (N) applications at panicle initiation were investigated for Zn biofortification in rice.*

Materials and methods: *Rice (cv. Super Basmati) seedling were grown in pots having a calcareous soil. All combinations of four Zn (control, Soil 6 mg Zn kg*$^{-1}$*, foliar 2 × 0.2% Zn and soil + foliar Zn) and three N (control, soil 20 mg N kg*$^{-1}$ *and foliar 0.5% N) levels were imposed at panicle initiation. At maturity, grains analysed for Zn and proteins.*

Results: *Grain protein concentration was significantly increased with foliar Zn treatments, and with soil and foliar N. Maximum grain Zn concertation (30 mg kg*$^{-1}$*) was achieved with application of soil Zn + foliar Zn + foliar N. At each N level, Zn application by either method significantly increased grain Zn concentration over control. This increase in grain Zn concertation at N levels was 36 to 54% with soil Zn + foliar Zn, 27 to 45% with foliar Zn and 9 to 15% with soil Zn over its control level.*

Conclusions: *Grain Zn concentration was significantly increased with soil N when combined with soil Zn, and with foliar N when combined with foliar Zn treatments. Conclusively, foliar N combined with soil + foliar Zn is the best combination of late Zn and N application for agronomic Zn biofortification in rice.*

INTRODUCTION

Lacking the affordability to a diversified food, people in many countries primarily rely on cereal grains to sustain their lives. Over time, intensive agriculture has depleted nutrients from soils *(1)* resulting in a poor nutritional quality of cereal grains produced from these soils. Moreover, the green revolution in the 1960s led to the development of high yielding cereal cultivars that have lower mineral density than old cultivars *(2, 3)*. As a consequence, people are encountering undernourishment of iron (Fe), zinc (Zn) and several other nutrients.

Zinc deficiency causes human health problems such as poor physical growth, weak immune system, low learning ability and deoxyribonucleic acid damage. Zinc biofortification of staple crops, both genetic and agronomic, is a promising approach and it has gained due perception by Consultative Group on International Agricultural Research *(4)*. Wheat, rice, and maize are important cereal crops that are the targets of Zn biofortification as being consumed mainly in countries having Zn deficiency in human populations. In rice grains, efforts are being made to increase the current baseline Zn level of 16 mg kg^{-1} to an acceptable level of 28 mg kg^{-1}.

Alkaline calcareous soils and flooded field conditions in the rice production systems in Asia decrease the availability of soil Zn to growing rice plants *(5)*. Not only the yield but also the quality of the produced grains is low under such production systems. Other factors contributing to low Zn availability from soil are low organic matter, high phosphate application and salt stress *(6)*. Agronomic biofortification by Zn application may simultaneously increase grain yield and grain Zn concentration in both standard and biofortified cultivars of cereals *(7)*. Zinc application by different methods significantly improved grain Zn concentration in rice *(8–10)*. Grain Zn concentration is enhanced more with foliar application of Zn during flowering and grain development stages than other methods of Zn application to the rice crop.

Zinc in grains is localized with proteins *(11)*. Application of N, both as basal and late dose, is known to increase root uptake, root-to-shoot translocation and remobilization of Zn *(12)*. For Zn biofortification in rice, combined applications of Zn and N to rice are more important than their sole applications *(13)*. However, it is still unknown if soil or foliar application of N at panicle initiation will be a better combination with different application methods of Zn to rice. The specific objectives of the experiment were to investigate: (i) the effect of late Zn and N applications on grain protein concentration and (ii) the best combination of Zn and N applications for Zn biofortification in rice.

MATERIAL AND METHODS

A pot experiment was conducted in a glasshouse at Department of Soil Science, Bahauddin Zakariya University, Multan (Pakistan). Soil for the study was collected from the surface layer (0–30 cm soil depth) of a field of at Agricultural Research Farm of the university. The soil was air-dried, crushed, and passed through a 2 mm sieve. A representative subsample of the soil was analysed for basic characteristics following standard methods *(14, 15)*. The clay loam (determined by Hydrometer method) had sand, silt, and clay contents of 26, 40 and 34%, respectively. The soil was alkaline calcareous in nature having pH 7.8 in saturated soil paste and 4% w/w acid neutralizable free $CaCO_3$. The electrical conductivity of soil saturated paste extract (EC_e), organic matter content and DTPA-extractable Zn of soil were 2.4 dS m^{-1}, 0.5% w/w, 0.7 mg kg^{-1}, respectively.

Each of 36 polyethylene lined plastic pot was filled in with 10 kg of the soil. Rice (cv. Super Basmati) seedlings (25 d old) were purchased from the local market. Three pairs of healthy seedlings of uniform height were transplanted in each pot in the last week of July 2016. A basal dose of nitrogen (25 mg kg^{-1} soil), phosphorus (25 mg kg^{-1} soil) and potassium (31 mg kg^{-1} soil) was applied as urea [$(NH_2)_2CO$] and potassium di-hydrogen phosphate (KH_2PO_4), respectively. After transplantation, water level in the pots was kept about 3 cm above the soil for the first 14 days, and then progressively increased to 5 cm height. For this purpose, tube-well water of good irrigation quality (pH 7.8, EC 0.34 dS m^{-1}, and undetectable Zn concentration) was used. All pots were randomized every 6th day to avoid the differential effects of microclimate in the glasshouse. Second and third splits of N, each of 25 mg kg^{-1} soil, were applied after 3 and 6 weeks of transplantation, respectively.

Experiment had twelve treatments in total, i.e. all possible combinations of four Zn levels (control, soil application at 6 mg Zn kg^{-1}, 2 foliar sprays of 0.2% w/v Zn solution and soil + foliar application) and three N levels (control, soil application at 20 mg N kg^{-1} and foliar spray of 0.5% w/v N) were applied at panicle emergence stage of rice. These fertiliser treatments were of urea [$(NH_2)_2CO$] and hydrated zinc sulphate ($ZnSO_4{\cdot}7H_2O$) and were applied additionally to the basal applications of macronutrient fertilisers. The treatments were arranged in completely randomized factorial design with three replications.

Irrigation was stopped a week before the harvest of the crop at maturity. Straws and panicles were cut and collected in separate paper bags. The plant samples were kept in an oven at 65 °C till constant weight and this was followed by the recording of straw and grains dry weights. Unhusked rice grains were dry ashed in a muffle furnace at 550°C followed by dissolution in 5 N hydrochloric acid (HCl) and dilution with distilled water *(16)*. Zinc in the digests was analysed on an atomic absorption spectrometer (Thermo Scientific 3000 Series, Waltham, MA, USA). For determination of N, separate samples of unhulled grains were digested on a hot plate with sulfuric acid (H_2SO_4) and hydrogen peroxide *(16)*. In wet-digested samples, a colour was developed with Nessler's reagent and absorbance was measured on a spectrophotometer (UV-1602, BMS, Quebec, Canada) 425 nm *(17)*. A factor of 5.7 was used to convert total N measurements to concentration of raw protein in grains.

Statistical significance ($P \leq 0.05$) of main and interactive effects of treatments on the recorded parameters was tested by two-way analyses of variance (ANOVA) test with interactions. The significant difference among measured was determined by Tukey's HSD test. All statistical analyses were carried out on SAS University Edition (SAS/STAT®, SAS Institute Inc., NC, USA).

RESULTS

Rice yield

Main effects of Zn and N significantly ($P \leq 0.05$) influenced grain and straw yield of rice (Table 1). As compared to Zn-control, grain and straw yields were increased by Zn application to the soil (Table 2). With soil Zn alone and Soil Zn + foliar Zn, grain yield increased respectively by 7 and 9% while that of straw yield by about 7%. Foliar-applied Zn did not affect straw or grain yield.

As compared to control level of N, grain and straw yields were increased by about 5% each with foliar-applied N and by 11 and 9%, respectively with soil-applied N (Table 2). In contrast to straw yield, grain yield was more with soil application of N than its foliar application.

Grain protein

Main effects of both Zn and N significantly ($P \le 0.05$) influenced concentration and contents of raw protein in grains (Table 1). However, variance in concentration and contents of grain protein was contributed more by N than Zn application.

Soil application of Zn did not affect grain protein concertation while foliar application alone or in combination with soil Zn increased grain protein concertation by 4%

Table 1. *Outcome (F values) of two-way analysis of variance (ANOVA) test*

Parameter	Source of variation		
	Zn	N	Zn × N
Grain yield	3*	11*	1
Straw yield	3*	9*	1
Grain protein concentration	4*	53*	1
Grain protein contents	5**	37*	1
Grain Zn concentration	161*	19*	6*
Grain Zn contents	65*	22*	2

Asterisk (*) denotes significant effect at $P \le 0.05$.

Table 2. *Yield response of rice grown in pots and fertilised, at panicle emergence stage, with all combinations of four zinc (Zn) levels and three nitrogen (N) levels*

Treatment levels	Grain yield (g pot^{-1})	Straw yield (g pot^{-1})
Main effect of Zn levels		
Control Zn	5.5±0.4 B	16.1±1.2 B
Soil Zn (6 mg Zn kg^{-1})	5.9±0.2 A	17.0±0.8 A
Foliar Zn (0.2% w/v Zn)	5.7±0.4 AB	16.5±1.2 AB
Soil Zn (6 mg Zn kg^{-1}) + Foliar Zn (0.2% w/v Zn)	6.0±0.5 A	17.1±0.9 A
Main effect of N levels		
Control N	5.5±0.3 C	15.9±0.9 B
Soil N (20 mg kg^{-1} soil)	6.1±0.3 A	17.4±0.7 A
Foliar N (0.5% w/v N)	5.8±0.4 B	16.7±1.2 A

Means ± standard deviations; Separately for each main effect, different letters indicate significant (P≤0.05) differences based on Tukey's HSD.

Table 3. *Concentration and contents of raw proteins in grains of rice grown in pots and fertilised, at panicle emergence stage, with all combinations of four zinc (Zn) levels and three nitrogen (N) levels*

Treatment levels	Grain protein concertation (g kg^{-1})	Grain protein content (mg pot^{-1})
Main effect of Zn levels		
Control Zn	76±5 B	423±53 B
Soil Zn (6 mg Zn kg^{-1})	77±5 AB	457±43 A
Foliar Zn (0.2% Zn w/v)	79±4 A	455±43 A
Soil Zn (6 mg Zn kg^{-1}) + Foliar Zn (0.2% Zn w/v)	79±4 A	469±54 A
Main effect of N levels		
Control N	73±3 B	399±28 C
Soil N (20 mg kg^{-1} soil)	80±2 A	489±29 A
Foliar N (0.5% N w/v)	80±2 A	464±38 B

Means ± standard deviations; Separately for each main effect, different letters indicate significant (P≤0.05) differences based on Tukey's HSD.

each over control level of Zn (Table 3). Zinc application, by any method, increased grain protein contents over control level of Zn; the increase ranged from 8 to 11%. The differences in both concentration and contents of grain protein were non-significant ($P \le 0.05$) at three applied levels of Zn.

Nitrogen application by either method significantly ($P \le 0.05$) increased concentration (by about 11% each with soil and foliar application of N) and contents (by about 23 and 16% respectively with soil and foliar-applied N) of protein in grains (Table 3). The contents of protein in grains were significantly ($P \le 0.05$) less with soil-applied N than its foliar application.

Grain zinc

Grain Zn concertation ranged from 19 to 30 mg kg^{-1} among twelve treatments comprising of all combinations of four Zn and three N levels (Figure 1). Grain Zn concentration was significantly ($P \le 0.05$) influenced by main and interactive effects of N and Zn applications at panicle initiation (Table 1). At each level of N, application of Zn by any method significantly ($P \le 0.05$) increased grain Zn concentration over control Zn (Figure 1). Similarly, at each level of N, the maximum increase in grain Zn concertation was achieved with combined soil + foliar Zn (36 to 54%) followed by foliar Zn alone (27 to 45%) and then soil Zn alone (9 to 15%).

At control level of Zn, the application of N, either to soil or on foliage, had a non-significant effect on grain Zn concertation over control level of N (Figure 1). Soil N increased grain Zn concentration over control level of N only when combined with soil Zn application. Foliar N

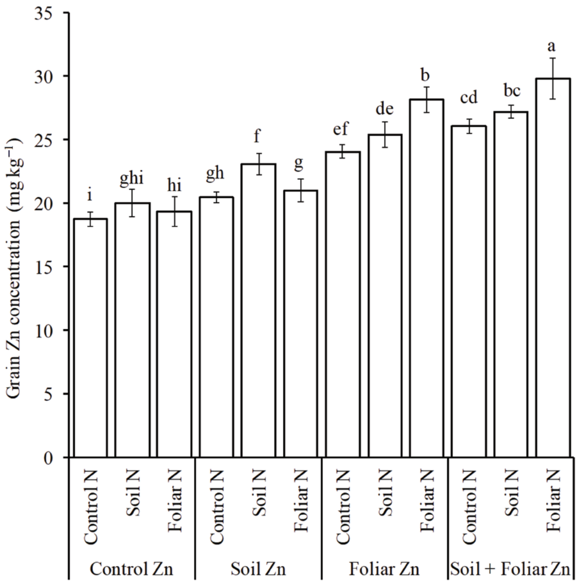

Figure 1. *Concentration of zinc (Zn) in grains of rice grown in pots and fertilised, at panicle emergence stage, with all possible combinations of four Zn levels [control, soil application (6 mg Zn kg^{-1}), foliar spray (2 × 0.2% w/v Zn) and soil + foliar application] and three nitrogen (N) levels [control, soil application (20 mg N kg^{-1}) and foliar spray (0.5% w/v N)]. Error bars are of standard deviations. Different letters on the bars indicate significant ($P \leq 0.05$) differences based on Tukey's HSD.*

increased grain Zn concentration over control level of N only when combined with treatments having foliar Zn application. Maximum grain Zn concertation was achieved with foliar N application to pots supplied with combined soil + foliar Zn applications.

Only main effects of N and Zn were significant ($P \leq 0.05$) for grain Zn contents (Table 4). As compared to control level of Zn, application of Zn by either method increased grain Zn contents producing maximum value with soil + foliar-applied Zn. Similar to grain Zn concentration, Zn contents were mainly increased by the treatments having foliar Zn applications; an increase of 38 and 53% respectively was recorded by foliar alone and soil + foliar applications of Zn over control level of Zn.

DISCUSSION

Soil Zn application, and both soil and foliar N applications at panicle initiation significantly ($P \leq 0.05$) increased straw and grain yield of rice (Table 2). This is because plants require a continuous supply of most of the nutrients throughout their life cycle. The level of yield response by a crop, however, is dependent on the rate, source, method and time of fertilizer application *(18)*. In the present study, Zn and N were applied at panicle initiation on 50% of main-tillers. During treatment application, therefore, many subtillers might have been in vegetative growth. A growth response was therefore expected especially due to the reason that soil was low in plant-available Zn and previously applied N might have already used by plant or lost in the environment. Moreover, N application at panicle initiation stage of rice increases grain yield by increasing grain weight in inferior spikelets *(19)*. However, application of N at late vegetative stage delays maturity and increases chances of lodging *(20)*. Therefore, field optimisation of rate and exact time of N application must to studied for economic yield returns along with quality grains.

Efforts have been done to increase protein and mineral densities in rice grains by conventional plant breeding and genetic engineering. Along with environmental protection and higher yields, agronomic fertiliser management is also meant to produce nutritious plant-based foods. For example, N fertilisation increased Zn and protein contents in rice grains along with an increase in grain yield *(21)*. In the present study, soil N fertilisation at panicle initiation also played a positive role in increasing grain protein concentration and contents in rice (Table 3). Application of N to cereals also increases grain accumulation of Zn by an increased sink in the form of water-insoluble proteins *(22, 23)*. Other reasons for increased grain Zn concentration by N application include increased uptake by the intensified root system, and better translocation and remobilisation of Zn towards grains *(24, 25)*. Therefore, a suitable quantity of N fertilizer at the flowering stage of rice is an important measure to obtain higher grain protein and grain Zn concentration.

Similar to N, the application of Zn also increases protein and Zn concentration in rice grains. Soil application, seed priming and foliar application are recommended to both flooded and direct seeded rice for this purpose *(9)*.

Table 4. *Contents of zinc (Zn) in grains of rice grown in pots and fertilised, at panicle emergence stage, with all possible combinations of four Zn levels and three nitrogen (N) levels*

Treatment levels	Grain zinc content (mg pot^{-1})
Main effect of Zn levels	
Control Zn	108±12 D
Soil Zn (6 mg Zn kg^{-1})	128±10 C
Foliar Zn (0.2% Zn w/v)	149±15 B
Soil Zn (6 mg Zn kg^{-1}) + Foliar Zn (0.2% Zn w/v)	165±19 A
Main effect of N levels	
Control N	123±19 B
Soil N (20 mg kg^{-1} soil)	146±22 A
Foliar N (0.5% N w/v)	142±31 A

Means ± standard deviations; Separately for each main effect, different letters indicate significant ($P \leq 0.05$) differences based on Tukey's HSD.

Zinc influences several physiological functions like synthesis, integrity and functioning of proteins in plants *(26)*. Moreover, Zn application might influence the uptake and translocation of N in rice plants *(13)*. Therefore, Zn application increased concentration and contents of protein in rice grains (Table 4).

From soil-applied Zn fertiliser, soil Zn^{2+} ion rapidly forms precipitates of zinc carbonate ($ZnCO_3$) under alkaline calcareous soils *(27)*. Therefore, recovery of soil-applied Zn is limited in high pH calcareous soils. Moreover, the availability of applied Zn decreases over time *(28)*. Therefore, late applications of Zn, especially in foliar form, are ideal for efficient uptake by plants. Late soil applications are also justifiable as a major fraction of Zn in grains is as a result of Zn uptake by roots after flowering stage *(29)*. Soil Zn application combined two foliar Zn applications at flowering are suggested by researchers for effective Zn biofortification in rice *(13, 30)*. Foliar Zn sprays are important as Zn is not fixed in soils and it has to travel little from leaf to grain as comparison to the soil where it travels from roots to grain *(31)*.

In the present study, soil N increased grain Zn concentration if combined soil Zn and foliar N application increased grain Zn concentration if combined with treatments having foliar Zn (Figure 1). Zinc translocate through xylem as Zn-phytosiderophore or Zn-amino acid and in phloem as Zn-nicotinamine complexes *(32)*. These organic compounds have N as their integral part. For N fertilized soils, therefore, the cotransport of Zn with N seems important for increased grain Zn accumulation by N application.

CONCLUSIONS

At panicle initiation, application of Zn and N increased concentrations of protein and Zn in rice grains. Results suggested that grain Zn concentration is significantly ($P \leq 0.05$) increased over respective control when both Zn and N were combined to soil or on foliage. This suggests that the role of N in Zn uptake, absorption, translation, and remobilisation is important for cotransport of Zn with N. Foliar + soil Zn when combed with foliar N increased grain Zn concertation to an acceptable level of Zn biofortification in rice.

ACKNOWLEDGEMENTS

The study was supported by Bahauddin Zakariya University, Multan (Pakistan).

DECLARATION

Authors declare no competing interests.

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